Expression of the Myelomonocytic Antigens CD36 and L1 by Keratinocytes in Squamous Intraepithelial Lesions of the Cervix

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The keratinocytes in squamous intraepithelial lesions (SILs) of the cervix show altered expression of a number of molecules involved both in the control of growth and differentiation and in cell surface interactions, particularly with components of the immune system. We have used tissue biopsies and in vitro model systems to investigate the expression in SILs of the molecules CD36 and L1, which are predominantly expressed by myelomonocytic cells but which also have functional roles in keratinocyte biology. Whereas the L1 protein (defined by the monoclonal antibody Mac387) was expressed by suprabasal and superficial cells in 12 of 12 cases of normal cervix (NCx) and in 14 of 14 cases of low-grade SILs (LG-SILs), in two of 16 cases of high-grade SILs (HG-SILs) it was entirely absent and in the remainder it was restricted to the most superficial layers. When an arbitrary grading scale was applied, L1 expression in HG-SILs proved to be significantly lower than in LG-SILs ($P < .01$) or in cases of NCx ($P < .01$). CD36 was expressed by superficial cells in four of 12 cases of NCx, in six of 14 LG-SILs, and none of 16 cases of HG-SILs (when graded, LG-SILs *v* HG-SILs = $P < .05$). The mechanisms underlying the expression of both molecules were investigated by growth in organotypic tissue culture of normal ectocervical epithelium and the cervical keratinocyte cell lines W12 (a model for LG-SILs) and CaSki and SiHa (models for HG-SILs). L1 was diffusely expressed by NCx cells and the W12 cell line, although its expression in the CaSki and SiHa cell lines was much more irregular and restricted. CD36 was occasionally present on the surface of superficial NCx and W12 cells, but was absent from CaSki and SiHa cells. Neither molecule could be induced by treatment of the cells with interferon-gamma. These data suggest that the expression of CD36 and L1 by cervical keratinocytes is related to their differentiation status rather than representing an effect of exogenous factors, such as those released by the immune cell infiltrate associated with SILs. CD36 may function as an immunoregulatory molecule on cervical keratinocytes in SILs, while L1 is more likely to be involved in the intracellular regulation of cell proliferation and maturation. Hum Pathol 25:73–79. Copyright © 1994 by W.B. Saunders Company

The human papillomaviruses (HPVs) have been strongly implicated in the development of squamous cell carcinoma of the uterine cervix[1] and of the precursor squamous intraepithelial lesions (SILs), which may be of low or high grade according to the degree of proliferative activity and loss of differentiation of the epithelial cells.[2] The fate of ectocervical epithelium after infection with HPV appears to depend not only on intracellular events controlling the capacity for proliferation and differentiation,[3] but also on extracellular interactions, particularly with components of the immune system.[4] There have been several reports of alterations in the numbers of immune cells in cervical disease, including Langerhans' cells,[5] lymphocytes,[6] and macrophages,[7] although the mechanisms for these changes are not known. It is becoming increasingly clear, however, that the neoplastic ectocervical keratinocytes may play an important role in modulating the local immune cell repertoire. These cells show altered expression of a number of immunocompetent molecules, including MHC class I,[8] MHC class II,[9] and adhesion molecules such as ICAM1.[10]

The molecules L1 and CD36 are predominantly expressed by leukocytes but also are potentially of importance in the intracellular and extracellular functioning of keratinocytes. There has been considerable interest in the expression of these molecules by squamous epithelium in a variety of sites and several studies have documented altered expression by keratinocytes in a number of pathologic conditions. CD36 is a multifunctional molecule predominantly expressed by platelets, monocytes, macrophages, and some endothelial cells.[11,12] It acts as a receptor for the extracellular matrix protein thrombospondin,[13] has a role in signal transduction, and is potentially important in certain immune interactions, particularly those involving immunologic accessory cells.[14] The L1 antigen, referred to inter alia as calprotectin,[15] is predominantly expressed by monocytes and neutrophils in the peripheral blood[16] and in the tissues by eosinophils and a subset of reactive macrophages.[17] As well as being implicated as a component of the innate defence system, there is evidence that the molecule also may serve to regulate epithelial cell proliferation and differentiation (reviewed by Brandtzaeg et al[15]). We report the expression of CD36 and L1 by keratinocytes in cases of normal and diseased cervix. We have observed alterations in the patterns of expression of both molecules in high-grade SILs (HG-SILs) and have used in vitro model systems to explore the mechanisms by which these changes arise.

MATERIALS AND METHODS

Specimens

Cervical punch biopsy specimens were taken from patients with abnormal colposcopic appearances and/or recent evidence of abnormal cervical cytology. Age-matched females undergoing laparoscopic sterilization were used as controls; these women showed no evidence of ongoing or previous cervical disease. All biopsy specimens were immediately mounted in OCT compound (BDH Ltd, Poole, UK) and snap-frozen in liquid nitrogen before being stored for up to 6 months in

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liquid nitrogen prior to use. Serial 6-μm frozen sections were cut, and routine haematoxylin-eosin staining was performed on every eighth level. On the basis of the hematoxylin-eosin staining the biopsy specimens were classified as histologically normal (n = 12) or as representing low-grade SILs (LG-SILs) (n = 14) or HG-SILs (n = 16). The morphologic changes were equivalent to those of koilocytosis and/or CIN1 in the LG-SILs and to CIN2 and CIN3 in the HG-SILs. After morphologic evaluation the remainder of the tissue cut from each section underwent immunohistologic assessment.

Immunohistology

The primary monoclonal antibodies used were OKM5 (Ortho Ltd; mouse IgG1, diluted 1:30) and SM0 (Serotec Ltd: mouse IgM, diluted 1:20) against CD36, and Mac387 (Dako Ltd; mouse IgG1, diluted 1:200) against L1. Each of the anti-CD36 antibodies gave identical staining results. Immunoperoxidase staining was performed using standard protocols, with avidin-biotin amplification and visualization of peroxidase activity with diaminobenzidine.

We devised an arbitrary grading scale to obtain a semiquantitative representation of antigen expression in the biopsy specimens. A total score was awarded for each tissue section, which represented the sum of the intensity and the extent of staining. The two parameters were scored as follows:

- Intensity: 0, no staining; 1, weak staining; 2, moderate staining; and 3, intense staining.
- Distribution: 0, occasional positivity; 1, patchy positivity; and 2, widespread positivity.

The results from each group of biopsy specimens were compared using the Wilcoxon rank sum test. Probability values, when given, are two-sided.

Cell Culture

Normal cervical epithelium (NCx) was cultured from uteri removed for noncervical disease. All patients had recently documented normal cervical smears and sections of uncultured cervical tissue appeared normal histologically. Cell culture was performed using protocols previously described.[18] Briefly, a sheet of ectocervical epithelium, which included the squamocolumnar junction, was finely minced before being stirred for 30 minutes at 37°C in a solution of 0.25% trypsin/0.01% EDTA in phosphate-buffered saline (PBS) to produce a single-cell suspension. The cells were grown with support from lethally irradiated Swiss G3T3 mouse fibroblast feeder cells and were maintained in Glasgow's modification of Eagle's medium (GMEM), supplemented with 10% (vol/vol) fetal calf serum (SeraLabs Ltd), 0.1 μg/mL hydrocortisone, and 10^{-10} mol/L cholera toxin (both Sigma Ltd). Colony formation was established by adding epidermal growth factor (Sigma Ltd) at 10 ng/mL 24 hours after plating. At subconfluence the feeders were removed with 0.01% EDTA in PBS and the keratinocytes were dislodged with 0.1% trypsin/0.01% EDTA in PBS before seeding onto collagen gels to establish them in organotypic culture (see below).

The cervical keratinocyte cell line W12 was derived from a cervical wart and contains HPV 16 DNA, predominantly in the episomal form, with approximately 100 copies/cell.[19] The cells are nontumorigenic in nude mice and can reform an epithelium that resembles LG-SILs. The W12 cell line is a unique in vitro model for the early stages of HPV 16-related cervical disease. The cells were grown with Swiss G3T3 support using protocols identical to those described for NCx cells before being established in organotypic culture.

The SiHa[20] and CaSki[21] cell lines were each derived from a squamous cell carcinoma of the cervix. Each is a tumorigenic cervical keratinocyte line containing integrated HPV 16 DNA, with one and 300 to 500 copies/cell, respectively. Prior to organotypic culture the cells were grown without feeder support in GMEM supplemented with 10% (vol/vol) fetal calf serum (SeraLabs Ltd).

Organotypic "Raft" Culture

The "raft" system of organotypic tissue culture allows cells growing on a collagen "gel" to differentiate at an air to liquid interface and enables them to produce stratified epithelia resembling the original lesions from which they were established. Eight milliliters of Vitrogen 100 collagen (Collagen Corp Ltd) were mixed on ice with 1.0 mL of 10× Vitrogen PBS solution and 1.0 mL of 0.1 mol/L NaOH. Two milliliters of the gel solution were then layered onto 5×10^5 lethally irradiated Swiss G3T3 fibroblasts in a 35-mm tissue culture dish, and gelation was initiated by warming to 37°C for 2 hours followed by equilibration with 2.0 mL of GMEM at 37°C overnight. The next day 10^6 keratinocytes were applied to the surface of each gel and maintained until confluent in GMEM supplemented with 10% fetal calf serum and cholera toxin. The collagen was then raised onto a metal grid such that the cells were able to differentiate at an air to liquid interface. After a further 10 days the collagen "rafts" were removed and cut into strips, which were mounted in OCT compound and snap-frozen in liquid nitrogen. Six-micrometer frozen sections were cut and immunohistochemical analysis was performed as described for the tissue biopsy specimens.

RESULTS

Staining of Tissue Biopsy Specimens

Normal Cervix. In all biopsy specimens from normal ectocervix the keratinocytes showed cytoplasmic expression of the L1 protein (Table 1). This was present in the suprabasal and superficial layers, with sparing of the basal cells (Fig 1, top left). CD36 was expressed on the surface of keratinocytes in four of the 12 specimens examined. The staining was generally patchy and weak, and was most prominent in the superficial layers, where it produced a net-like pattern (Fig 1, bottom left), although some basal positivity also was observed occasionally. There was no spatial association between CD36+ keratinocytes and infiltrating immune cells.

Low-grade Squamous Intraepithelial Lesions. L1 expression was seen in the cytoplasm of keratinocytes in all 14 of the LG-SILs examined. Its distribution within the epithelium was similar to that in normal ectocervix and included expression by koilocytes (Fig 1, top center). In six of the 14 biopsy specimens keratinocytes showed surface expression of CD36 (Fig 1, bottom center). In some specimens the expression was patchy and weak, similar to that in normal ectocervix, although in others it was stronger and more extensive. Again, there was no correlation between CD36+ keratinocytes and infiltrating immune cells.

High-grade Squamous Intraepithelial Lesions. Reduced expression of L1 was seen in HG-SILs. The molecule could not be detected in two of the 16 biopsy specimens examined and in the majority of the remainder it was restricted to the most superficial layers (Fig 1, top right). In general, L1 was detectable in areas of cellular differentiation and was absent from the mor-

TABLE 1. Semiquantitative Representation of CD56 and L1 Expression in Cervical Biopsy Specimens

Biopsy	Antigen					
	CD56			L1		
	NCx	LG-SILs	HG-SILs	NCx	LG-SILs	HG-SILs
No. of biopsy specimens examined	12	14	16	12	14	16
No. of positive biopsy specimens	4	6	0	12	14	14
Mean score	0.42‡	0.71*	0	4.00†	3.79†	2.31
Median score	0	0	0	4	4	2
Range of scores	0-2	0-3	0	3-5	2-5	0-4

Note: An arbitrary grading scale was used (see Materials and Methods section). *Statistical analysis:* Differences between high-grade lesions and low-grade lesions or normal cervix. * $P < .05$; † $P < .01$; ‡not significant. There was no significant difference between low-grade lesions and normal cervix for either molecule (Wilcoxon rank sum test; P values are two-sided).

phologically most atypical cells. There was no expression of CD56 by keratinocytes in any of the HG-SILs (Fig 1, bottom right).

An arbitrary grading scale was used to quantitate the expression of CD56 and L1 by cervical ke[redacted]inocytes in each of the three groups (Table 1). The expression of CD56 in the LG-SILs was significantly greater than that in the HG-SILs ($P < .05$), although there was no significant difference between the LG-SILs or HG-SILs, and cases of NCx. L1 expression in HG-SILs was significantly less than that in the LG-SILs ($P < .01$) or cases of NCx ($P < .01$), but there was no significant difference in L1 expression between the LG-SILs and cases of NCx.

Staining of Cell Lines

The mechanisms underlying the expression of CD56 and L1 in vivo were explored using cell culture techniques. When the cervical keratinocytes were grown in monolayer culture no expression of CD56 and L1 (as determined by flow cytometry) was observed in any case (data not shown). Cell stratification and maturation were induced using the "raft" technique of organotypic tissue culture, and the cytokine-mediated inducibility of the molecules on each cell type was quantified by culturing the cells in the presence of recombinant interferon-gamma.

Normal cervical cells produced a differentiating epithelium up to 10 cell layers thick. L1 was expressed by all cells except those in the basal layers, and was cytoplasmic in distribution (Fig 2, top left). CD56 was expressed infrequently and only by superficial and differentiated cells (arrowed cell, Fig 2, top right).

W12 produced a somewhat thinner epithelium showing disorganization of the basal layer but with surface differentiation consistent with a low-grade cervical lesion. There was strong cytoplasmic expression of L1 by the suprabasal and superficial cells (Fig 2, center left). Basal cells were generally negative, although there was some focal expression. CD56 was expressed on the surface of superficial, differentiated cells (Fig 2, center right), including some that were vacuolated and that resembled koilocytes.

CaSki cells produced an epithelial layer of up to 12 cells. This appeared disorganized and highly atypical throughout its full thickness, reminiscent of a high-grade cervical lesion. There was mild irregularity of the base of the epithelium, but no unequivocal infiltration of the subjacent collagen. L1 showed a restricted pattern of expression, with many areas entirely negative for the molecule. Patchy positivity was observed in some places, however, and this occurred throughout the full thickness of the epithelium (Fig 2, bottom left). CD56 was not seen in any of the sections examined (Fig 2, bottom right). SiHa cells also produced an atypical epithelial layer, and showed patterns of expression of both molecules that were essentially the same as those seen on CaSki cells (data not shown).

All cell types were grown in organotypic culture in the presence of recombinant interferon-gamma. Different doses (150 and 300 U/mL) were used, and culture was maintained for 24, 48, and 72 hours prior to sectioning. However, no detectable modulation of either molecule was seen on any of the four cell types examined.

DISCUSSION

The mechanisms underlying the development of intraepithelial neoplasia in the ectocervix are complex. The abnormal keratinocytes show a number of alterations in molecules likely to be involved in intracellular signalling and control of proliferation[5,22,23] as well as in interactions with cells and molecules in the extracellular environment.[8,9,24] CD56 and L1 have important roles in leukocyte biology[12-15] and may contribute to intracellular and extracellular interactions of keratinocytes at a number of sites.[25-27] The expression and modulation of these molecules in the cervix have not been investigated previously, however.

The distribution of CD56 in cases of NCx is consistent with some reports of its localization in the skin,[27,28] although the factors controlling expression of the molecule by cervical keratinocytes are unclear. Whereas interferon-gamma has been shown to upregulate CD56 on normal epidermal keratinocytes in vitro,[29] an observation supported by evidence of increased CD56 expression on keratinocytes in inflamed skin,[30,31] this cytokine is unlikely to contribute significantly to the expression seen in the cervix. We saw no association

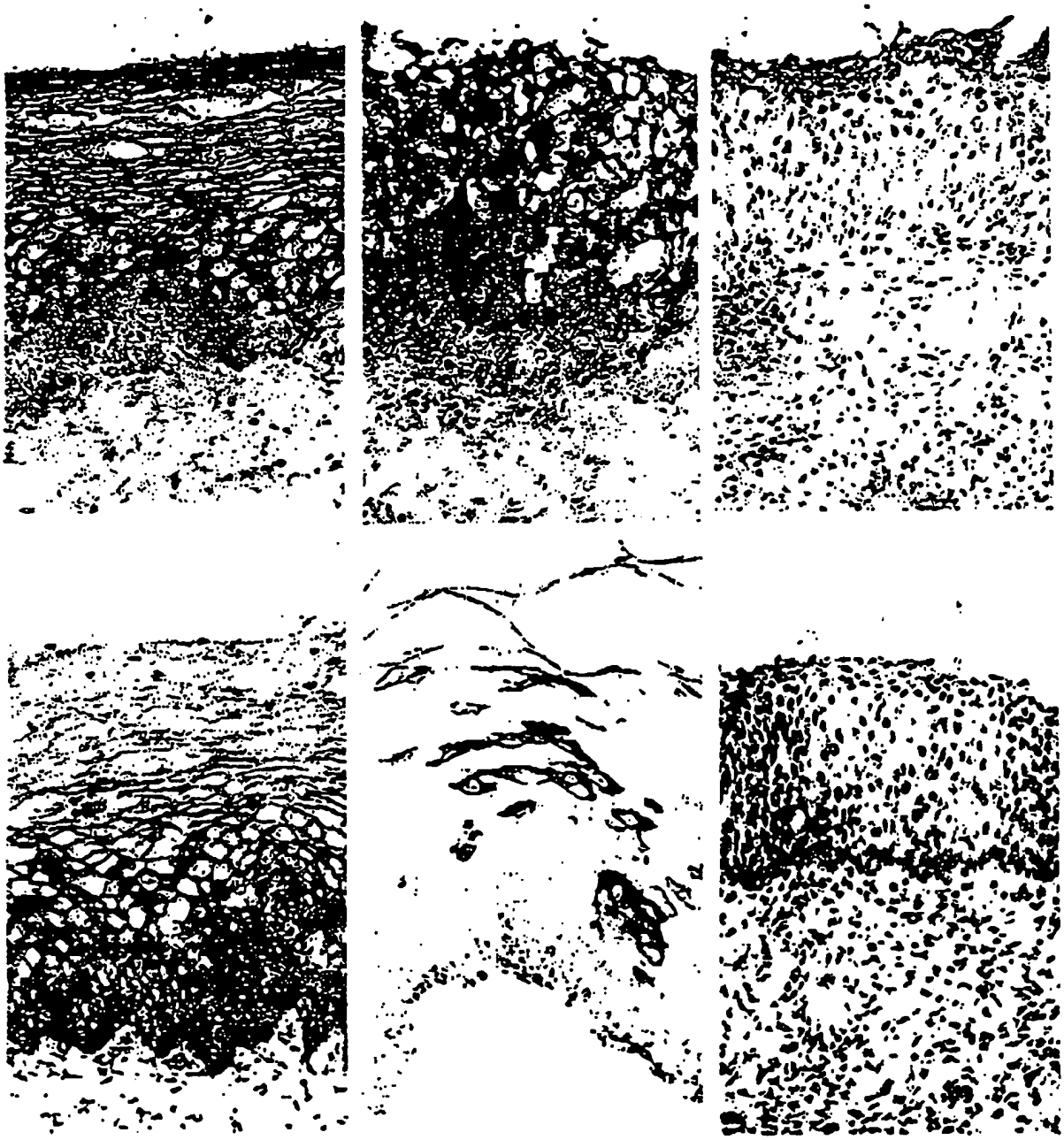

FIGURE 1. Expression of L1 (top) and CD36 (bottom) on keratinocytes in cases of NCx (top left and bottom left), LG-SILs (top center and bottom center), and HG-SILs (top right and bottom right). See text for details. (Immunoperoxidase staining using avidin-biotin amplification and diaminobenzidine as chromogen; magnifications ×160.)

between CD36 positivity and numbers of lymphocytes in the epithelium or subepithelial stroma of any of the cervical biopsy specimens we examined, and recombinant interferon-gamma did not modulate CD36 expression on the cervical keratinocytes in tissue culture. It is more likely that the presence of CD36 is related to the maturational state of the cervical keratinocytes, as has been suggested for epidermal cells.[32] This hypoth-

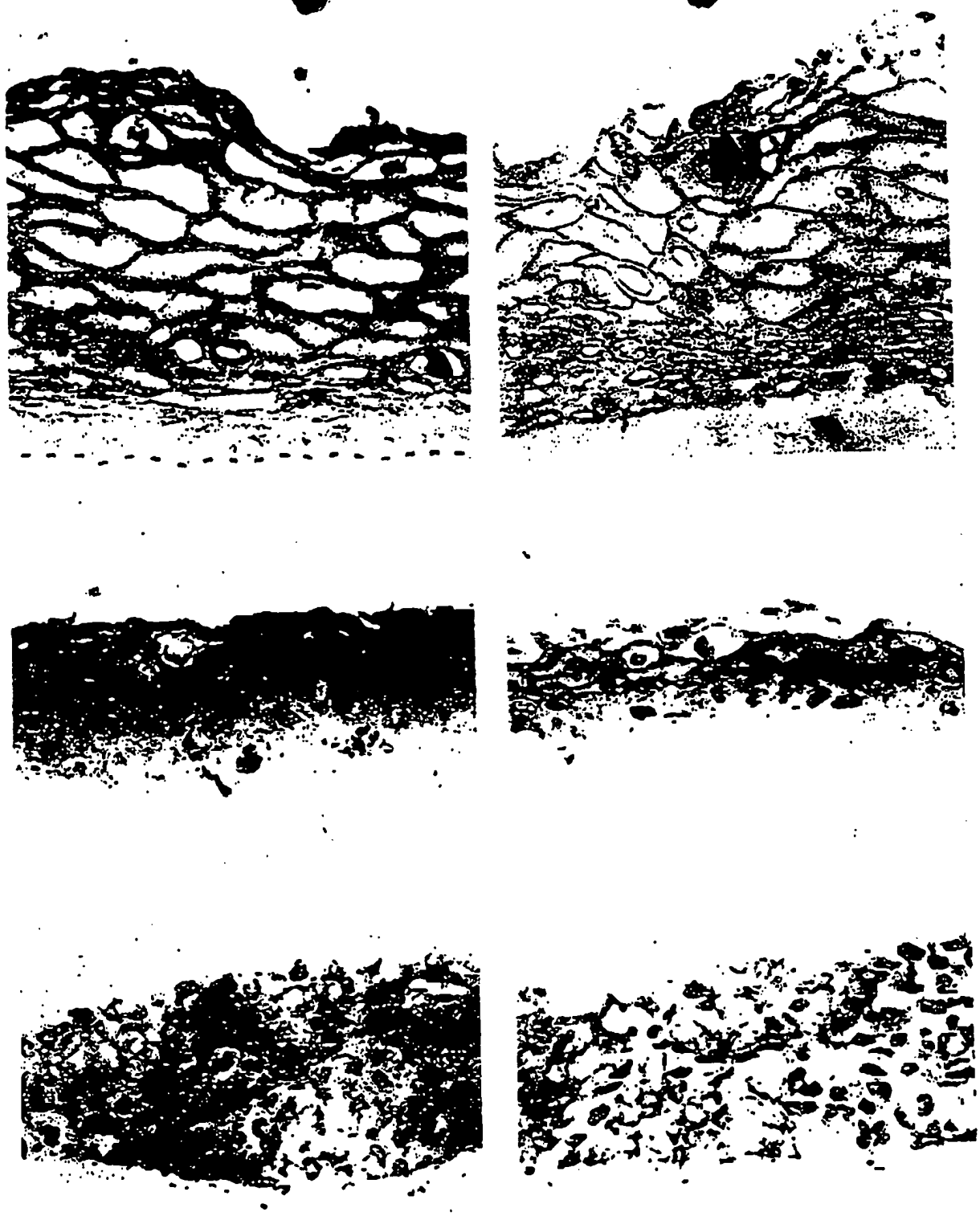

FIGURE 2. Expression of L1 and CD36 by cervical keratinocytes in organotypic tissue culture: top left and right, NCx; center left and right, W12; bottom left and right, CaSki. Top left, center left, bottom left: L1; top right, center right, bottom right: CD36. (Immunoperoxidase staining using avidin-biotin amplification and diaminobenzidine as chromogen. The base of the epithelium is indicated by a dashed line in the top left panel. Magnifications x320.)

esis is supported by the constitutive expression of CD36 in differentiated layers of W12 and NCx cells, with no expression in monolayer culture, where the capacity for differentiation is restricted, and no expression by CaSki and SiHa cells, which display a limited differentiation program even in organotypic tissue culture. Expression by differentiated keratinocytes may be related to activation of intracellular signalling pathways or to autocrine or paracrine stimulation from cytokines released on differentiation. Either of such pathways may be modulated as part of the cytopathic effects of HPV on superficial keratinocytes in low-grade cervical lesions, although this has not been investigated in vitro.

The functional role of CD36 expression by cervical keratinocytes remains unclear. The molecule has been claimed to contribute to lymphocyte:keratinocyte adhesion in the epidermis,[12,13] but we do not believe that CD36 plays a significant role in the initial interactions required for T-cell trafficking into the ectocervix, which involves binding of lymphocytes to basal keratinocytes. Monoclonal antibodies to CD36 produced no reduction in lymphocyte binding to cervical keratinocytes in monolayer culture (data not shown), whereas antibodies to ICAM1 were able to reduce binding by 50%.[10] It remains conceivable, however, that CD36 has a role in lymphocyte retention in the superficial ectocervical epithelium, where CD36 is expressed in some cases in vivo.

CD36 on keratinocytes also may function as an immunologic accessory molecule, similar to its role on CD36+, HLA-DR+, CD11b− monocytes.[14] As well as being able to present soluble antigen, these cells are able to activate autologous T cells in the absence of added antigens or mitogens, and such an autologous mixed lymphocyte reaction is associated with the development of suppression of immune responsiveness.[54,55] The CD36+ keratinocytes in cases of NCx and in the LG-SILs did not express CD11b (data not shown) and it is conceivable that they may act in an immunoinhibitory capacity in these lesions. HLA-DR is expressed by keratinocytes in some LG-SILs (Coleman et al, unpublished observation), but we saw no CD36+, HLA-DR+ keratinocytes in the 14 cases we studied. Nevertheless, as CD36+ cells are present in LG-SILs the induction of any local immune response may induce the CD36+, HLA-DR+, CD11b− phenotype, which may then serve to suppress the reaction before any amplification phase can ensue.

Our results with biopsy specimens from cases of NCx confirm that L1, a major constituent of myelomonocytic cells, also is expressed by keratinocytes of normal mucosal squamous epithelia.[25] The preservation of staining in LG-SILs, with reduced expression in HG-SILs, is consistent with findings from studies of squamous neoplasia at other anatomic sites.[56-58] Such differential expression patterns on cervical keratinocytes in vivo are mirrored by the in vitro findings that L1 is only patchily present on the high-grade epithelium produced by CaSki and SiHa cells, but is strongly expressed on differentiated layers of NCx and W12 cells. Our observations suggest that, as for CD36, the pattern of expression of L1 by cervical keratinocytes is most likely to be related to their differentiation status rather than representing an effect of exogenous factors, with lack of expression in HG-SILs reflecting the relative lack of differentiation of the neoplastic squamous epithelial cells.

Several functions have been ascribed to the L1 protein. It shows antimicrobial activity at biologic levels in vitro[59,60] and may be active against organisms that enter the cytosol. The antiviral properties of L1 have not been investigated, but it is conceivable that the molecule may function at least partly in the defence of cells, such as keratinocytes against viruses, including HPVs. Furthermore, L1 is a member of the growing family of S-100 proteins, which appear to be involved in cell cycle progression and cell differentiation,[61] and there is evidence that it can inhibit the proliferation of a number of transformed and nontransformed cell lines, possibly by inhibition of casein kinase II.[62] The absence of immunodetectable L1 from high-grade cervical disease therefore may not only be a morphologic correlate of restricted keratinocyte differentiation, but it also may represent a demonstrable intracellular event of importance in the neoplastic progression of such lesions.

We have presented evidence that normal ectocervical keratinocytes express both the L1 and CD36 antigens, albeit at different frequencies. The expression of both molecules is maintained in LG-SILs, in which significant infiltration by host immune cells is absent,[3] but is lost in HG-SILs, which are associated with an immune cell response.[6] Our in vitro data suggest that this loss of expression is more likely to result from changes intrinsic to the keratinocyte rather than representing the effect of external factors, such as those derived from infiltrating cells. The functional implications of these observations is unclear, but the pattern of expression we have observed may reflect alterations in intracellular signalling pathways contributing to the neoplastic process, as well as changes likely to influence the interactions of cervical keratinocytes with immune cells and possibly with intracellular infectious agents.

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